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Three SIBLINGs (Small Integrin-Binding LIgand, *N*-linked Glycoproteins) Enhance Factor H's Cofactor Activity Enabling MCP-like Cellular Evasion of Complement-mediated Attack*

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Previously we have shown that two members of the newly named SIBLING (small integrin-binding ligand, *N*-linked glycoproteins) family of proteins, bone sialoprotein, and osteopontin, bound first to a cell surface receptor and then to complement Factor H thereby blocking the lytic activity of the alternative pathway of complement. Another member of this family, dentin matrix protein 1, is shown in this paper to be very similar to osteopontin in that it can bind strongly to Factor H (K_a ~ 1 nM) and block the lytic activity through either the vitronectin receptor ($\alpha_V\beta_3$ integrin) or CD44. Binding of Factor H to SIBLING localized to the cells surface was demonstrated by fluorescence-activated cell sorting. Extensive overlapping fragment analyses suggests that both dentin matrix protein 1 and osteopontin interact with cell surface CD44 through their amino termini. Similar fragments of bone sialoprotein, like the intact protein, did not functionally interact with CD44. All three proteins are shown to act in conjunction with Factor I, a serum protease that, when complexed to appropriate cofactors, stops the lytic pathway by digesting the bound C3b in a series of proteolytic steps. These results show that at least three members of this family confer membrane cofactor protein-like activity (MCP or CD46) upon cells expressing RGD-binding integrins or CD44. The required order of the assembly of the complex suggests that this cofactor activity is limited to short diffusional distances.

Recently we have proposed that a number of proteins whose genes are clustered together on human chromosome 4 (mouse chromosome 5) are a genetically related family termed SIBLINGs,[1] for small integrin-binding ligand, *N*-linked glycoproteins. While direct comparisons of the primary protein sequences of these proteins would not lead to a hypothesis that these proteins are closely related, a systematic look at the: 1) properties of each exon (containing casein kinase II phosphorylation sites or Arg-Gly-Asp (RGD) integrin-binding tripeptide, polyacidic stretches, etc.); 2) the exons involved in splice variants (identical exons) and; 3) the fact that all introns interrupt the coding sequences only between codons, clearly suggests that these clustered genes are related (1). At this time, the gene products within this family include four acidic proteins: bone sialoprotein (BSP) (2), osteopontin (OPN) (3), dentin matrix protein 1 (DMP1) (4), and dentin sialophosphoprotein (5, 6). Matrix extracellular protein (7) (also know as OF45 (8)) is a positively charged protein that appears to be a more distantly related member of the SIBLING family. Bone acidic glycoprotein-75 (9) may be a member due to several of its biochemical and biological properties but it has not yet been cloned and sequenced.

Except for a high affinity for hydroxy apatite among the acidic members and a universal ability to support cell attachment *in vitro* (through their RGD integrin-binding tripeptides), very little has been described about the possible common shared functions of the SIBLING proteins. Recently we have shown that both BSP and OPN can protect cells from being lysed by the alternative complement pathway (ACP) (10). Both proteins strongly bound in a stoichiometric fashion to complement Factor H, the major humoral protein that controls ACP. Furthermore, it was shown that to have this protective effect, the SIBLING must first bind to the vitronectin receptor (for both) or CD44 (for OPN) and then to Factor H for the complex to inhibit the lysis of the cells. Whenever the SIBLING-Factor H complex was allowed to form before binding to the cell's surface receptor(s) the protective properties were lost. This loss of activity appeared to be due to a masking of cell receptor-binding sites by the preformed SIBLING-Factor H complexes. This observation suggests that the functional range of the secreted BSP or OPN is likely to be limited to the distance that they can diffuse to a cell surface receptor before being bound and inactivated by the relatively abundant Factor H in the blood and tissue fluids.

DMP1 was first cloned from a rat cDNA library by George *et al.* (11) and was shown to have an acidic primary structure as well as numerous phosphorylation sites. The integrin-binding tripeptide, RGD, first observed in the rat cDNA sequence and confirmed in many species since, has been shown to support cell attachment by some cells *in vitro* (12). Although it was first proposed to be dentin specific, the message for DMP1 has been identified in a number of other mineralized tissues as well as brain (6, 13). In 1997, Hirst *et al.* (14) published the genomic organization of the human DMP1 gene and excluded the locus from a causative role in at least two families with dentinogenesis imperfecta type II (14).

In this paper, we will show that this third SIBLING protein,

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[1] The abbreviations used are: SIBLING, small integrin-binding ligand *N*-linked glycoprotein; RGD, arginine-glycine-aspartate; BSP, bone sialoprotein; OPN, osteopontin; DMP1, dentin matrix protein 1; MCP, membrane cofactor protein; ACP, alternative complement pathway; MEL, murine erythroleukemia; GVB, gelatin veronal buffer; MTT, thiazolyl blue; DAF, decay accelerating factor; SEC, size exclusion chromatography; FACS, fluorescence-activated cell sorting; ELISA, enzyme-linked immunsorbent assay; PBS, phosphate-buffered saline.

DMP1, can also protect tumor cells from attack by the ACP and does so by bridging Factor H to integrins and CD44. Furthermore, the structural similarities and differences between the SIBLING family members are exploited to investigate the mechanism and sequences involved in complement modulation.

EXPERIMENTAL PROCEDURES

Reagents—Rabbit anti-DMP1 peptide-derived antibody, LF-148, was raised against the human sequences (C)EHPSRKIFRKSRISE and (C)LKNIEIESRKLTVDAYH conjugated through the Cys to activated horseshoe crab hemocyanin (Pierce Chemical Co., Chicago, IL). This antiserum bound to fragments D6 and D8 (see Fig. 5 under "Results") in direct ELISA suggesting that both peptides successfully raised useful IgG components. Furthermore, this antiserum recognized recombinant mouse DMP1 made in *Escherichia coli* and full-length bovine DMP1 described below. Normal human serum, purified human complement Factor H protein, and mouse monoclonal antibody against Factor H were obtained from Quidel Corp. (San Diego, CA). A monoclonal antibody against human complement Factor I that blocks Factor I activity (cleavage of C3b) (catalog number A247) as well as a monoclonal antibody to Factor I that binds but does not block Factor I function (catalog number A231) were also obtained from Quidel Corp. Polyclonal antibodies against CD-44 and a "functional" antibody against $\alpha_V\beta_3$ (catalog number MAB1976) were obtained from Chemicon Co. (Temecula, CA). Synthetic purified glycine-arginine-aspartate-serine peptide (GRGDS) was obtained from Calbiochem-NovaBiochem Corp. (La Jolla, CA). Synthetic peptides corresponding to the sequences VKQADSGSSEEKQ (OPN exon 3) and LYNKYPDAVATWLNPDPSQKQNLLAPQ (OPN exon 4) were made and purified by the Peptide Laboratory of the Facility for Biotechnology Resources, Center for Biologics Evaluation and Research, Food and Drug Administration (Bethesda, MD). Preimmune serum, human serum-adsorbed goat anti-rabbit IgG conjugated to horseradish peroxidase (1 mg/ml IgG) as well as goat anti-mouse IgG conjugated to horseradish peroxidase (1 mg/ml IgG) were obtained from Kirkegaard & Perry (Gaithersburg, MD). Dulbecco's modified essential medium, Hank's balanced salt solution, and heat inactivated fetal bovine serum were obtained from BioFluids, Inc. (Rockville, MD).

Western Blotting—Samples diluted in gel sample buffer were resolved by Tris glycine SDS-PAGE 4–20% gradient gels (Novex Corp., San Diego, CA) and transferred to nitrocellulose following standard conditions (15). Nitrocellulose membranes were rinsed with Tris-buffered saline (0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl) containing 0.05% Tween 20 (TBS-Tween). After a 1-h incubation in blocking solution (TBS-Tween + 5% non-fat powdered milk) at room temperature on rotary shaker, primary antibody was added (1:2,000) and incubated overnight at 4 °C. The nitrocellulose sheet was washed in TBS-Tween four times for 5 min each time with TBS-Tween and then horseradish peroxidase-conjugated second antibody (1:50,000) in TBS-Tween + 5% milk was added and incubated for 2 h at room temperature. Following removal of the second antibody solution the membrane was washed three times with TBS-Tween and rinsed a final time in enzyme substrate buffer for 5 min. Enhanced chemiluminescence reagents were employed for signal detection (Pierce Chemical Co., Chicago, IL) with x-ray film.

High Performance Liquid Chromatography—A Shimadzu LC10AS binary gradient system was employed for chromatographic separations. Size exclusion chromatography utilized a 1.0 × 30-cm Superose 6 column (Amersham Bioscience, Piscataway, NJ) equilibrated in 0.05 M sodium phosphate, pH 7.4, containing 50% fresh formamide at a flow rate of 0.5 ml/min. The column was calibrated using commercially available protein standards of known molecular weight (Amersham Bioscience).

Direct ELISA—Greiner high-binding 96-well plates (part number 655061) were coated with 100 μl of high performance liquid chromatography fractions overnight at 4 °C. Plates were washed three times (5 min each) with TBS-Tween and exposed to 100 μl of 1:2000 primary antibody for 1 h at room temperature. Plates were washed three times and exposed to 100 μl of 1:2000 horseradish peroxidase-conjugated goat anti-rabbit IgG. Following a 1-h incubation at room temperature, plates were washed again three times with TBS-Tween and color was developed using 3,3′,5,5′-tetramethylbenzidine and H_2O_2 for 10 min at room temperature. Color development was stopped by the addition of 25 μl of 1 N H_2SO_4 and analyzed at 450 nm.

Production of Recombinant Intact SIBLINGs—Recombinant human BSP and OPN were made and expressed as described previously (10). For DMP1 expression, an adenoviral construct was generated by subcloning full-length bovine DMP1 cDNA (6, 13) into high expression, replication-deficient adenovirus (Ad5) using the cytomegalovirus promoter. The construct was selected, purified, and expressed following the method described previously for BSP and OPN adenoviruses (10). Briefly, adenovirus was plaque-selected and propagated on HEK 293 cells (ATCC number CRL1573). Viral particles were purified by twice banding on CsCl and viral titers evaluated by plaque formation of virus dilutions on HEK293 cells (16). Recombinant DMP1 was generated by infecting subconfluent normal human marrow stromal fibroblasts with 10,000 pfu/cell. Harvested serum-free media was subjected to anion exchange chromatography. Native BSP, DMP1, and OPN proteins were purified by diluting medium from normal human marrow stromal fibroblast cells 1:1 with 40 mM phosphate buffer, pH 7.4, and loading directly on a 5.0 × 2.0-cm column packed with ToyoPearl TSK QAE resin. A linear salt gradient to 2.0 M NaCl was employed to separately purify the three proteins to ~95% purity as measured by SDS-PAGE.

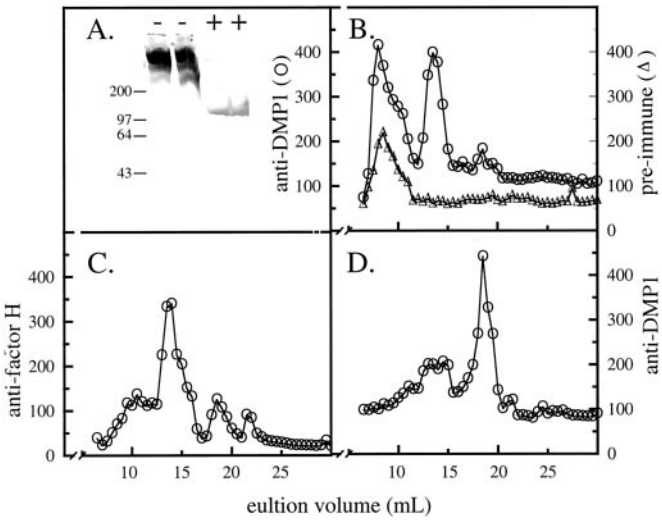

FIG. 1. **DMP1 in human serum.** *A*, aliquots of normal human serum ± heating and reduction with dithiothreitol were analyzed by SDS-polyacrylamide gradient gel electrophoresis. Proteins were transferred to nitrocellulose and probed with anti-DMP1 antibody LF-148. *B*, unreduced normal human serum was fractionated by SEC and aliquots of fractions from the SEC were transferred to 96-well microtiter plates and analyzed by direct ELISA for DMP1 (○). The DMP1 activity eluted at a location consistent with that in a complex with Factor H. Substituting non-immune rabbit serum for the LF-148 antibody yielded immunoreactive material corresponding to the void peak (△) showing that it is nonspecific. *C*, aliquots of the same column profile were analyzed by direct ELISA for Factor H immunoreactivity using a monoclonal antibody. *D*, serum heated at 100 °C and reduced with 2 mM dithiothreitol for 10 min prior to SEC analysis was also analyzed for anti-DMP1 immunoreactivity. The activity eluted at the expected location of free DMP1.

Production of Recombinant SIBLING Fragments—A pET-15b vector (Novagen Inc., Madison, WI) which produces polypeptides as fusion products with an amino-terminal polyhistidine sequence followed by a thrombin cleavage site (MGSSHHHHHHSSGLVPRGSH) was used for expression and generation of most of the peptides. Peptides were purified from isopropyl-1-thio-β-D-galactopyranoside-induced log phase *E. coli* by nickel affinity chromatography (Ni^{2+} IMAC) following the manufacturer's protocol. Sequences of primers used to insert the in-frame appropriate restriction sites (*Nde*I and *Bam*HI) and generate the relevant SIBLING fragment by PCR for insertion into the vector are given. All OPN fragments were derived from the human sequence (3). The two OPN fragments containing the RGD domain (O4 and O5) were engineered in a pET-22b vector with *Nco*I sites because of an internal *Nde*I site. The use of pET-22b results in fusion polypeptides with the six His residues at the carboxyl terminus. The first two DMP1 peptides (D1 and D2) were made using the bovine DMP1 cDNA as template (13) and all others were made using human genomic DNA for the PCR templates.

Alternative Complement Mediated Cell Lysis Assay—Murine erythroleukemia (MEL) cells (a gift of Dr. Marilyn Farquhar, University of California, San Diego, CA) grown in Dulbecco's modified essential medium containing 10% fetal bovine serum and 4 mM glutamine were rinsed three times with gelatin veronal buffer (GVB, Sigma) containing 2 mM Mg^{2+} and 8 mM EGTA. Cells were resuspended in GVB-MgEGTA

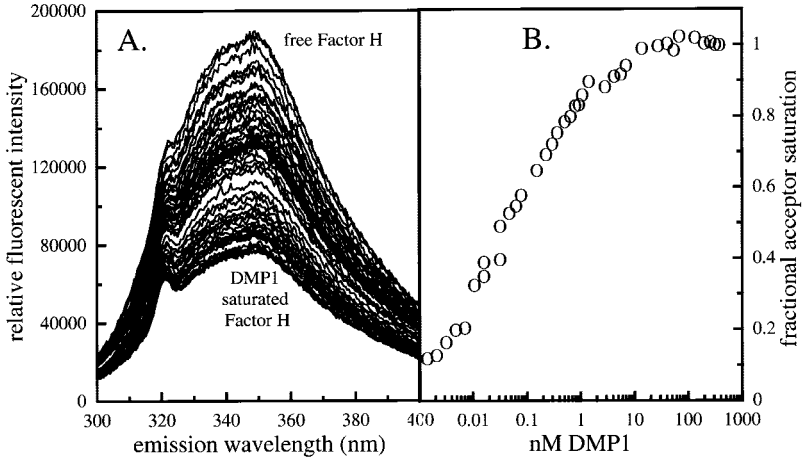

FIG. 2. **Fluorescence titration.** *A*, intrinsic tryptophan fluorescence was monitored by excitation at 295 nm and emission from 300 to 500 nm using a Photon Technology International Series M fluorimeter. The initial Factor H concentration was 28 nM and DMP1 was added in nanomolar amounts. Both Factor H and DMP1 were dissolved in Hank's balanced salt solution. *B*, the binding curve was determined following calculation of fractional acceptor saturation. Factor H contains 25 tryptophan while DMP1 contains two. Given the abundance of tryptophans in Factor H and the molecule's robust fluorescent emission signal, the contribution of the fluorescent spectra arising from DMP1 was negligible. The fluorescent signal for Factor H was progressively quenched until an equimolar amount of DMP1 was added showing a 1:1 saturable binding. The estimated binding of DMP1 to Factor H is in the nanomolar range.

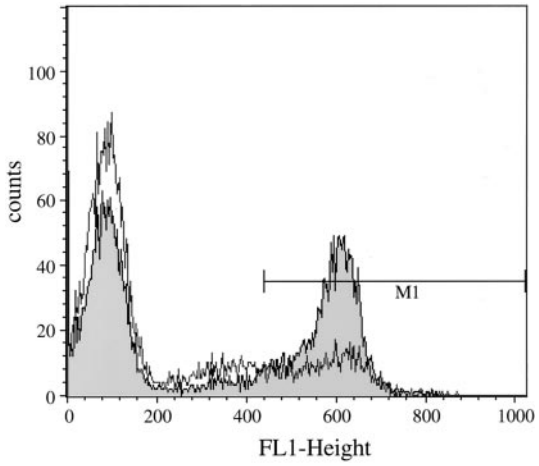

FIG. 3. **Demonstration of DMP1-factor H binding by FACS analysis.** MEL cells treated as described under "Experimental Procedures" incubated with either PBS or recombinant DMP1 for 10 min at room temperature. The cells were then washed twice and incubated with Alexa Fluor 488-labeled complement protein factor H for 10 min at room temperature. The cells were washed twice, re-suspended in PBS, and then analyzed by FACSCalibur cell sorter equipped with a 488-nm argon laser using Cellquest software. *Shaded area* under the curve marks the observed profile for the DMP1 + Factor H-treated cells. Only those cells pretreated with the DMP1 bound significant amounts of labeled Factor H.

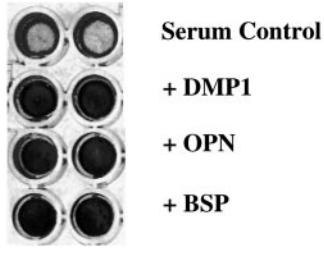

FIG. 4. **SIBLINGs confer protection from complement-mediated lysis.** MEL cells are lysed by the ACP in human serum and this results in a failure to produce the blue color from MTT (Serum Control). MEL cells in GVB-MgEGTA buffer were treated with 10 μg/ml DMP1, OPN, or BSP prior to the addition of normal human serum diluted 1:10 in GVB-MgEGTA. Following a 2-h incubation, cell viability was monitored by the thiazolyl blue assay. Tissue culture well images were captured using a Polaroid DMC Ie digital microscope camera.

(gelatin veronal buffer containing 2 mM magnesium and 8 mM EGTA) at a density of 5×10^6 cells/ml. Cells were preincubated with 10 μg of DMP1, BSP, or OPN in 1 ml for 10 min at 37 °C followed by incubation at 37 °C with normal human serum diluted 1:10 in GVB-MgEGTA. After 2 h, cells were harvested for thiazolyl blue viability assay by incubating a 50-μl aliquot of the cell suspension in an equal volume of 1 mg/ml thiazolyl blue (MTT) for 45 min. Cell viability was determined spectrophotometrically by absorbance at 560 nm.

Fluorescence-activated Cell Sorting (FACS) Analysis—MEL cells (2×10^6/ml) were washed twice in PBS and incubated with either PBS or recombinant DMP1 (10 μg/ml) for 10 min at room temperature. The cells were then washed twice and incubated with 5 μg/ml Alexa Fluor 488 (Molecular probes, Eugene, OR) -labeled purified human complement protein Factor H (Quidel, San Diego, CA) for 10 min at room temperature. The cells were washed twice, re-suspended in PBS, and then analyzed by FACSCalibur cell sorter equipped with a 488-nm argon laser using Cellquest software (BD PharMingen, Bedford, MA).

RESULTS

DMP1 Exists as a Complex in Serum Bound to Factor H—We have proposed that, based on a shared chromosomal localization, similarities in exon structure and intron type, that DMP1 belongs to the SIBLING family of proteins (1). We have previously found that two other acidic SIBLING family members, BSP and OPN, are bound to complement Factor H in serum and that disruption of the complex requires heating and reduction (10). The strong (nM) interaction between Factor H and either BSP or OPN (which have no cysteine residues) is noncovalent and required reduction to disrupt due to the unique structure of Factor H. By NMR spectroscopy, BSP and OPN lack ordered structure and exist extended and flexible in solution (1), while Factor H is a large and highly structured protein containing 20 repeated short consensus repeat motifs. Each short consensus repeat contains 4 cysteine residues that are involved in intra-repeat disulfide bonds forming a "sushi roll"-type structure. The status of DMP1 in human serum was studied by SDS-PAGE and Western blotting as well as by size exclusion chromatography (SEC). When aliquots of normal human serum diluted 1:10 were subjected to SDS-PAGE followed by transfer to nitrocellulose and probing with a peptide-derived antibody against DMP1, immunoreactive bands were readily apparent (Fig. 1*A*). The migration position of immunoreactive DMP1 shifted upon heating and reduction consistent with the destruction of the DMP1-Factor H complex.

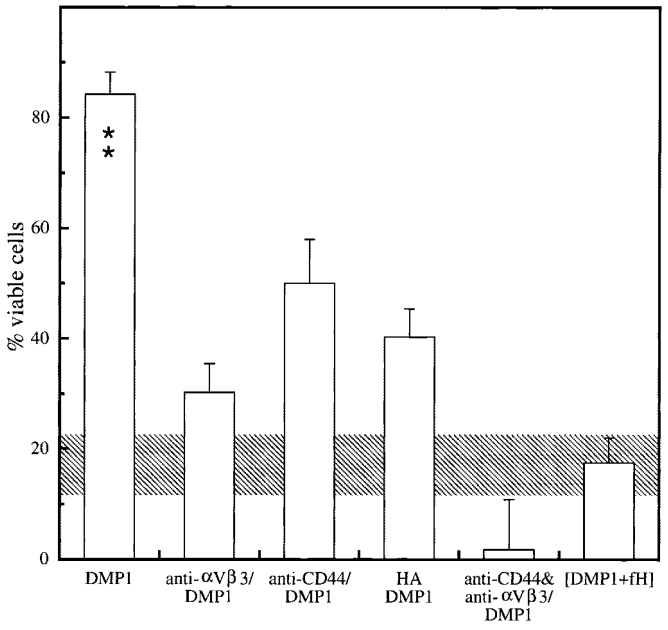

FIG. 5. **Integrin and CD44 involvement in DMP1-mediated protection from lysis by complement.** MEL cells in GVB-MgEGTA buffer were pretreated with either 10 μg of DMP1 (control, *first bar*), or a 1:4000 dilution of an $\alpha_V\beta_3$ antibody (*second bar*), a 1:4000 dilution of a CD44 antibody (*third bar*), 100 μg of hyaluronic acid (*fourth bar*) or a mixture of anti-CD44 and anti-$\alpha_V\beta_3$ each diluted 1:4000 (*fifth bar*). DMP1 (10 μg) was then added to all but the DMP1 control. Cell viability was determined by MTT assay after the addition of human serum to all samples. MEL cells were also incubated with a preformed complex of DMP1-Factor H (DMP1 + fH, *last bar*) to test whether Factor H binding to the SIBLING first (before subsequent association with the cell) altered the protection from complement-mediated lysis. The data represents the mean and S.E. for three separate experiments. Statistical significance was determined by analysis of variance (**, $p \leq 0.01$). Percent cell viability was determined using 560-nm absorbance values including a control where no serum had been added (100% viable). The *crosshatched region* represents that range of values observed when normal human serum (1:10) alone was added (maximal cell death). The results show that DMP1 must bind first to either integrins or CD44 before binding to Factor H to protect the cells from death by lysis.

Unreduced normal human serum was fractionated by size exclusion chromatography and aliquots of fractions from the SEC were transferred to 96-well microtiter plates and analyzed by direct ELISA for DMP1. Immunoreactive material was evident in two peaks, a void volume peak and an included peak. Incubation of aliquots from the same SEC fractions with preimmune serum (in place of anti-DMP1) yielded a single void volume peak. Thus the void volume peak appearing in the anti-DMP1 profile was most likely the result of nonspecific binding. Direct ELISA of the same fractions for Factor H immunoreactivity yielded a single peak that co-migrated with the included DMP1 immunoreactive material (Fig. 1*C*). SEC resolution of a separate aliquot of the same normal human serum that had been incubated with reducing agent and heat to dissociate the binding complex yielded an immunoreactive profile upon direct ELISA analysis that shifted to ~100 kDa, the location of authentic, free DMP1 (Fig. 1*D*). The immunoreactive material that eluted in the void volume in unreduced samples (Fig. 1*B*) was absent in the profile of reduced serum, suggesting that the epitope recognized by the antibody was sensitive to denaturation.

DMP1 and Factor H Binding—Complement Factor H possesses 25 tryptophan residues, while DMP1 contains 2. Thus, the binding between Factor H and DMP1 can be followed by intrinsic tryptophan fluorescence. Titration of purified human complement Factor H with DMP1 was followed by excitation at 295 nm and monitoring emission between 300 and 450 nm. The emission profile of Factor H alone yields a peak at 347 nm (Fig. 2*A*). The addition of DMP1 in nanomolar increments causes a relative fluorescent intensity quenching. Conversion of the fluorescent intensity titration into a binding curve by determining the fraction of binding sites occupied as the fractional change in fluorescence quenching at 347 nm yields a saturable binding curve (Fig. 2*B*). By steady state fluorescence, the binding of DMP1 by Factor H is saturable, possess a 1:1 stoichiometry, and has a binding constant in the nanomolar range. This value is similar to Factor H interactions with BSP and OPN reported earlier.

DMP1 Bridges Factor H to the Cell Surface—The direct binding of Factor H to DMP1, while DMP1 is engaged with its cell surface receptor was studied by fluorescence-activated cell sorting. MEL cells incubated with DMP1, briefly washed, incubated with Alexa Fluor 488-conjugated purified human complement protein Factor H, and then analyzed by FACSCalibur cell sorter. The results indicate that a significant association of cell surface Factor H was evident only in the DMP1-treated cells (Fig. 3). Similar shifts were seen when cells were subjected to an initial incubation with BSP and OPN (data not shown).

DMP1 Protects Cells from Alternative Complement Pathway-mediated Lysis—DMP1 binding to Factor H suggests that this SIBLING may also confer resistance to humoral complement surveillance. The ability of rDMP1 to protect cells from complement activity was investigated using the MEL cell line which, when incubated with normal human serum, can be readily assayed for ACP-mediated cell lysis (10, 17). Cell survival was measured by MTT reduction by living mitochondria. Titration with dilutions of normal human serum and time courses were carried out to define optimal incubation conditions. The addition of purified recombinant SIBLING to MEL cells followed by treatment with normal human serum protected the cells from lysis (Fig. 4). The protection of MEL cells from ACP-mediated cell lysis by DMP1 addition exhibited a dose response (data not shown).

CD44 and $\alpha_V\beta_3$ Are Involved in DMP1 Binding to the Cell Surface—The SIBLINGs, BSP, and OPN, were previously found to protect cells from complement-mediated lysis through an initial cell surface binding to a receptor. For BSP the membrane receptor was $\alpha_V\beta_3$ while OPN was found to interact with either $\alpha_V\beta_3$ or CD44 (10). The identity of the receptor(s) involved in DMP1 conferred protection from lysis was investigated (Fig. 5). Pretreatment of MEL cells with an anti-$\alpha_V\beta_3$ antibody that blocks ligand binding decreased the protective effect of DMP1, although some protective activity remained. Preincubation with anti-CD44 antibody as well as with hyaluronan, a natural ligand for CD44 (18), also reduced the protective effect of added DMP1. Thus, DMP1 behaves similarly to OPN, exhibiting $\alpha_V\beta_3$ as well as CD44 binding properties. When anti-$\alpha_V\beta_3$ and anti-CD44 antibodies were combined and preincubated with the cells, the protective effect of DMP1 was completely abolished. Treatment of MEL cells with a preformed complex of DMP1-Factor H also eliminated the DMP1's protective effect in complement-mediated lysis showing that DMP1 already complexed to Factor H can no longer bind to either its $\alpha_V\beta_3$ or CD44 receptor. These data are consistent with a model where SIBLINGs bound to factor H first, in solution, lack the ability to bind to their cell surface receptors.

DMP1- and OPN-CD44-Binding Domains—The structural similarities and differences of the SIBLING family members can be exploited to determine the sequences involved in specific binding interactions. A series of overlapping peptides for OPN and DMP1 were created to use in identifying binding sequences (Fig. 6). An *in vitro* assay for functional binding was designed

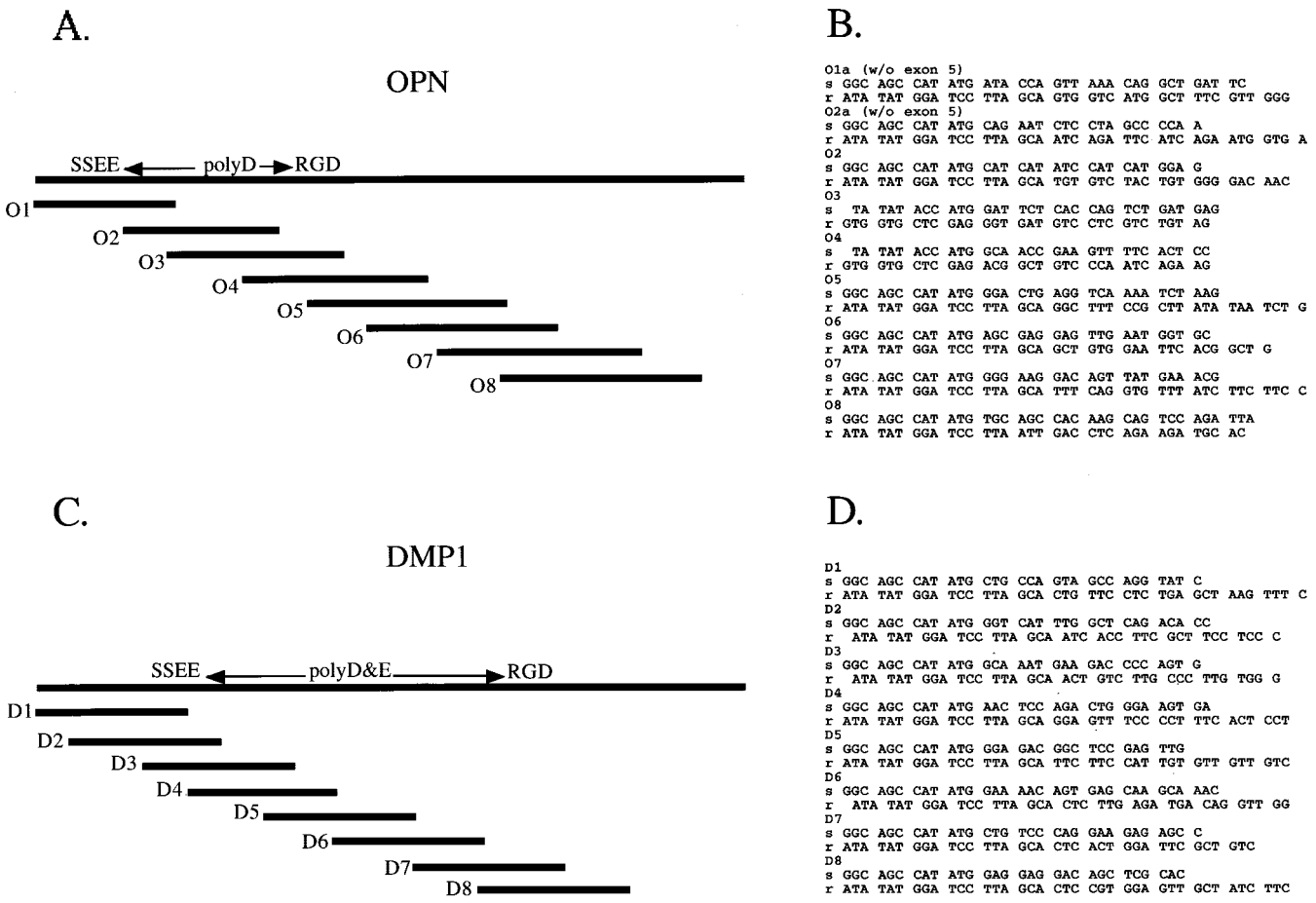

FIG. 6. **Overlapping fragments of OPN and DMP1 used for mapping CD44-binding domain.** Overlapping peptides of OPN (*A*) and DMP-1 (*C*) were constructed from the corresponding oligonucleotide pairs used to generate the PCR fragment for insertion into pET-15b *E. coli* expression vector (*B* and *D*). The first two fragments of DMP1 are derived from the bovine sequence, all others are human. Fragment 7 of DMP1 contains the RGD sequence. The two OPN fragments containing the RGD domain (*O4* and *O5*) were engineered in a pET-22b vector with *Nco*I sites because of an internal *Nde*I site. DMP1 is drawn at half the scale of OPN.

to test each fragment's ability to block intact SIBLING-conferred protection from lysis. First, the cell's $\alpha_V\beta_3$ integrins were blocked by saturation with GRGDS peptide so that any added OPN or DMP1 should interact only with CD44. Incubation of these $\alpha_V\beta_3$-blocked cells with DMP1 or OPN fragments was followed, first by treatment with intact DMP1 (or OPN), and then normal human serum. When the cells were assayed for viability by thiazolyl blue the domain(s) of DMP1 or OPN that interact with the CD44 protein on the surface of the MEL cells could be determined.

Treatment of MEL cells with normal human serum alone (−GRGDS, −SIBLING) resulted in significant cell death (Fig. 7*A*). Incubation of the cells with SIBLING (−GRGDS, +SIBLING) prior to the addition of normal human serum gave rise to cell protection. Pretreatment with GRGDS (+GRGDS, −SIBLING) alone had no effect on cell viability and was equivalent to control (−GRGDS, −SIBLING). Saturation with GRGDS did not entirely block the protective effect of OPN or DMP1 (+GRGDS +SIBLING), consistent with OPN and DMP1 binding to the alternate receptor, CD44.

To investigate the sequences involved in SIBLING binding to CD44, experiments were carried out where the $\alpha_V\beta_3$ integrin was first saturated by an RGD-containing peptide, thus, fragments will only block activity if they contain regions involved in CD44 binding. When GRGDS saturation was followed by incubation with the various OPN and DMP1 fragments, the amino-terminal region of both clearly blocked the SIBLING's normal protective effect by 80 to 90% (Fig. 7*B*). With the exception of the DMP1 carboxyl terminus, the other fragments had little or no effect. Thus, the amino terminus of both OPN and DMP1 appear to be the major sites of interaction with CD44. Furthermore, they are similar to each other and distinct from that of BSP, a SIBLING which does not interact with CD44 in this functional assay. The structural sequence involved in CD44 binding was further refined by the use of synthetic peptides in a competition assay as described above. The peptides corresponding to exons 3 and 4 from osteopontin were screened for the ability to abolish DMP1- or OPN-conferred protection from alternative complement mediated cell lysis. The peptide corresponding to VKQADSGSSEEKQ significantly reduced DMP1 and OPN-mediated protection (Fig. 7*C*). The sequences that successfully blocked the protection from ACP-mediated lysis conferred by OPN or DMP are listed in Table I. Of the peptides tested, fragment 1 from OPN and DMP1 as well as DMP1 fragment 8 reduced cell survival. A conserved potential binding sequence among all three peptides is illustrated in Table I.

SIBLING-Factor H Mechanism of Protection—Factor H exerts its regulatory action by two major pathways. When bound to certain proteins or carbohydrate groups, Factor H dissociates and inactivates the assembled C3 convertase at the cell surface, an action named decay accelerating activity typified by the membrane protein decay accelerating factor, or DAF (CD55). Factor H can also serve as an essential co-factor for cell surface-associated Factor I-mediated cleavage of C3b into a series of fragments. These fragments are unable to promote the lytic pathway but each has corresponding receptors on a num-

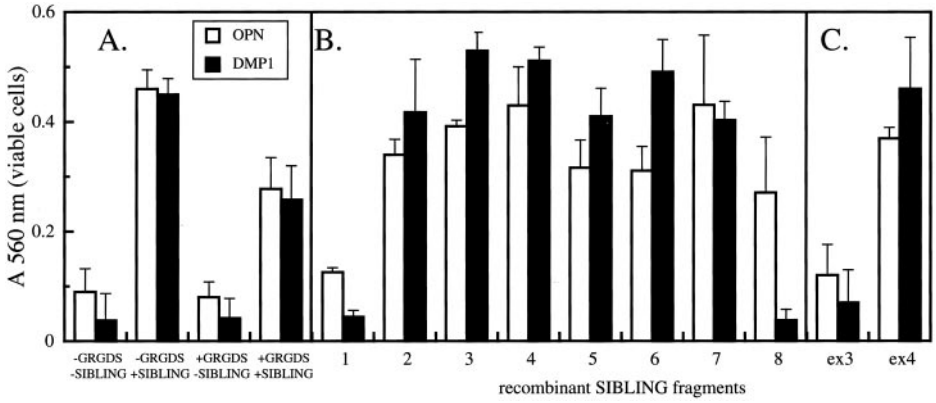

FIG. 7. **CD44 binding sequences within DMP1 and OPN are predominantly at the amino termini.** MEL cells were transferred into GVB-MgEGTA buffer and rinsed three times to remove residual fetal bovine serum in the growth medium. Control incubations consisted of a 1:10 dilution of normal human serum alone (−GRGDS −SIBLING), 5 μg of intact recombinant OPN or DMP1 followed by normal human serum (−GRGDS +SIBLING), 400 nM GRGDS followed by normal human serum (+GRGDS −SIBLING), and 400 nM GRGDS followed by 5 μg of intact recombinant OPN or DMP1 and then a 1:10 dilution of normal human serum (+GRGDS +SIBLING) (*panel A*). For the fragment studies, MEL cells in GVB-MgEGTA buffer were treated with 400 nM GRGDS to saturate the $\alpha_V\beta_3$ integrin, followed by an incubation separately with 8 different fragments of either OPN or DMP1 (*numbers 1–8*, at 10 μg/ml) and then followed by an incubation with 5 μg of intact recombinant OPN or DMP1 (*panel B*). For the peptide studies, MEL cells in GVB-MgEGTA buffer were treated with 400 nM GRGDS to saturate the $\alpha_V\beta_3$ integrin, followed by an incubation with 10 μg/ml OPN exon 3 or OPN exon 4 peptide and then followed by an incubation with 5 μg of intact recombinant OPN or DMP1 (*panel C*). All conditions were then treated with a 1:10 dilution of normal human serum and after 2 h the cells were analyzed for viability by the thiazolyl blue assay (as in Fig. 4). Two separate experiments were carried out and combined for the OPN fragments and OPN peptides, while three separate experiments were carried out for DMP1 fragments. The average of all experiments ± S.D. is shown.

TABLE I
SIBLING sequences involved in CD44 binding

The DMP1–1 fragment was engineered using the bovine cDNA as template for this aminoterminal region (13), while the DMP1–8 fragment was engineered using the human genomic DNA for PCR templates (4). For osteopontin, fragment 1 and exon 3 were engineered using the human sequence (3).

	Peptide sequence that blocks protective effect of intact SIBLING.
DMP1-1	LPVARYQNTESKSSEEWKGHLAQTPTPPLESSESSEESKLSSEEQ
DMP1-8	EEDSSHTLSHSKSESREEQADSESSESLNFSEESPESPEDENSSSQEGLQSHSSSAESQSEESHSEEDDSDSQDSSRSKEDSNSTE
OPN-1	IPVKQADSGSSEEKQLYNKYPDAVATWLNPDPSQKQNLLAPQTLPSKSNESHDH
Exon 3	VKQADSGSSEEKQ

ber of immune cells. To investigate which pathway is involved in the SIBLING/Factor H-mediated dampening of complement lytic activity, a series of experiments using the MEL cell line, recombinant adenovirus produced SIBLINGs and complement active normal human serum were carried out. In these experiments, the serum is pretreated with specific blocking antibodies prior to its addition to cells in the presence or absence of a given SIBLING. Two monoclonal antibodies against Factor I were used at different doses (1:4000, 1:2000, and 1:1000) in pretreating the human serum. One of these antibodies has been characterized as a "cleavage-blocking" antibody in that, when it is bound to Factor I, there is no cleavage of C3b. The other anti-Factor I antibody, when bound to Factor I, does not block C3b cleavage.

When DMP1 as well as OPN and BSP were assayed for Factor I involvement in SIBLING-conferred protection from complement, the cleavage blocking anti-Factor I antibody diminished the SIBLING's ability to protect the cells (Fig. 8*A*). In contrast, the other non-function blocking antibody did not significantly alter the SIBLING's ability to protect the cells. A dose response was evident in the ability of the cleavage-blocking antibody to inhibit cell protection by DMP1 (Fig. 8*B*). These results are consistent with a model where SIBLING-mediated protection from lysis involves Factor H action through Factor I. It is possible that some Factor H-mediated DAF activity is also present, however, it is insufficient by itself to stop ACP lysis. Thus, the (cell surface receptor ($\alpha_V\beta_3$ or CD44), -SIBLING-Factor H) complex acts similar to the membrane cofactor protein (MCP, CD46) in facilitating C3b degradation by binding and activating Factor I.

DISCUSSION

Complement plays a role in immune adherence, inflammation, opsonization, viral neutralization, localization of antigen, and cell lysis. The complement system can be activated by at least three distinct pathways: the classical pathway (usually involving immunoglobulins), the alternate pathway, and the lectin pathway (22). While the initiators of each pathway are different, all pathways converge in formation of the membrane-bound C3 convertases (Fig. 9). The different activation pathways employ different proteins to form the C3-convertases, however, all C3 convertases are multicomponent serine proteases that cleave the same single peptide bond in serum protein C3 generating two active fragments. A small peptide, C3a, is released as well as the major fragment C3b which can covalently attach to local targets. The newly bound C3b directs immune clearance, antigen selection, and cell lysis (19–22). C3b can also function via the ACP C3-convertase in an amplification loop generating more bioactive C3b. Thus, active complement results in the covalent attachment of a large number of C3b molecules clustered around the C3-convertase. C3b binding to C4b2b or C3bBb subunits of C3-convertase results in C5-convertase activity. C5-convertase acts on C5 generating C5a, a small peptide expressing anaphlatoxin and chemotaxin

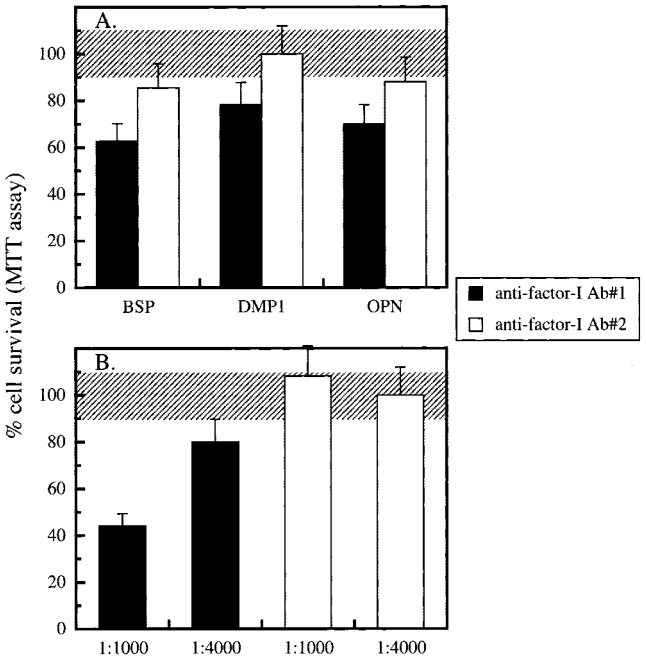

FIG. 8. **Factor I involvement in SIBLING protection from lysis by complement.** *A*, MEL cells suspended in GVB-MgEGTA were pretreated with 5 μg of SIBLING for 10 min. Complement active normal human serum (1:10 dilution in GVB-MgEGTA) was preincubated for 10 min with a 1:2000 dilution of a cleavage-blocking antibody (*Ab#1*) or an antibody that binds to Factor I but does not interfere with cleavage of C3b (*Ab#2*). This treated serum was added to the SIBLING-treated cells and survival was followed with MTT. Percent cell survival was calculated using control cohort cells that had not been treated with normal human serum. Notice that the cells are no longer protected if the Factor I is pre-bound by the inactivating antibody, clearly showing that the SIBLING protective affect is working though a mechanism similar to MCP. *B*, there is a dose-response for the protective affect of DMP1 by 1:1000 and 1:4000 dilutions of cleavage blocking antibody, Ab#1 but neither dose of the non-blocking antibody, Ab#2, had any affect on the cells survival.

activity, and C5b, a large fragment that initiates assembly of the membrane attack complex.

Complement is regulated by a family of proteins, termed regulators of complement activation. The family of proteins include complement receptors one (CR1, CD35) and two (CR2: CD21), DAF (CD55), membrane cofactor protein (MCP, CD46), as well as Factor H (23). There are two major mechanisms by which Factor H and its cofactors can disrupt the lytic portion of the ACP. Factor H, when induced into a proper conformation by binding to other proteins or to certain carbohydrate groups, can displace the Bb from the C3 convertase, C3bBb. This is called decay-accelerating activity. On many mammalian cells this activity is also associated with the membrane-associated protein, DAF (decay-accelerating factor, CD55). Dissociation of the convertase aborts the lytic pathway.

Alternatively, the modified serine protease, complement Factor I, can be bound and activated by a small number of different proteins leading to the cleavage of the C3b protein into a series of defined products. Each consecutive protease step results in a C3-product that can be recognized by different cell surface receptors. Factor I action on C3b generates C3bi which serves as a ligand for CR3 ($\alpha_M\beta_2$, CD18/CD11b) and CR4 (gp150, CD18/CD11c), two integrin-type receptors. Factor I can also further cleave C3bi into a released large fragment, C3c (a ligand for CR1) and a remaining covalently bound fragment C3dg (a ligand for CR2). These receptors are found in various combinations on immune cells including natural killer cells, monocytes, macrophages, B and T cells etc. and are used in the identification of cells that have been marked by the complement system for destruction. Factor I cleavage of sparse C3b molecules on cell surfaces or of fluid-phase C3b constitutes inactivation as C3b fragments from this cleavage cannot participate in the formation of C3 convertase. Factor H has weak cofactor activity that can be greatly enhanced by binding to various factors.

Using MEL cells and recombinant DMP1, we have found that, following interaction with a specific cell surface receptors ($\alpha_V\beta_3$ or CD44), DMP1 sequesters Factor H to the membrane phase and that this interaction quenches alternative complement-mediated cell lysis by normal human serum. Furthermore, DMP1 and OPN binding to CD44 was found to involve predominantly these two SIBLING's amino-terminal region and could be blocked by an amino-terminal peptide. Finally, the receptor-SIBLING-Factor H complex clearly used functional Factor I in order to protect cells from lysis. These results suggest a shared biological activity between the SIBLING family members BSP, OPN, and DMP1 in their ability to regulate complement activity through Factor I-mediated cleavage of C3b (Fig. 9*B*). In solution phase, SIBLING binding to Factor H yielded no protection from lysis, while Factor H binding to SIBLINGs previously associated with the membrane phase (*i.e.* bound to $\alpha_V\beta_3$ and/or CD44) resulted in protection from lysis. This biological activity of the SIBLINGs was found to include a MCP-like mechanism associated with the complement cofactor, Factor I. That all three SIBLING members studied, DMP1 (in the current study) and BSP and OPN (10) share Factor H binding suggests that common structural features are involved, such as polyacidic amino acid sequences and high sialic acid content. It is of note that the presence of sialic acid on a cell surface increases the affinity of C3b for factor H, which prevents the formation of C3-convertase (24, 25).

The complement dampening activity of these three SIBLINGs is negated whenever the proteins are bound first by the very abundant Factor H (~0.5 mg/ml in serum) prior to binding to the appropriate cell surface receptor(s). This raises the interesting point that the range of this complement dampening activity must be relatively short. It seems reasonable to assume that only the cell actually secreting the protein (autocrine) or possibly cells within a short diffusional distance (paracrine) can bind the SIBLINGs to their cell surfaces and be protected from local complement activity. Cells expressing SIBLING family members and the appropriate cell surface receptors would have the capacity to locally dampen the complement cascade. In the case of complement and immune adherence, SIBLING expression might confer an immunoprotected status. Inflammatory responses involving complement may also be subject to SIBLING modulation. Increased OPN levels have been observed in wounds (26, 27), while elevated BSP levels have been observed in arthritis (28–30). Up-regulation of regulators of complement activation proteins has been observed in inflammatory tissues and organs affected by autoimmune diseases, while expression of regulators of complement activation components by autologous cells undergoing apoptosis was decreased (31). Expression of SIBLINGs by neoplasms (as has been seen for BSP and OPN) may provide a "gain-of-function" in a selective survival advantage for tumor cells (10). This survival advantage involves the new ability of the cancer cell to subvert the immune/complement system of surveillance through SIBLING sequestration of complement Factor H to the tumor cell surface. The membrane phase SIBLING-Factor H complex then recruits Factor I in the cleavage and clearance of C3b, thereby dampening complement activity and cloaking the cells from surveillance. The biological significance of the serum SIBLING-Factor H complex could be that it scavenges free

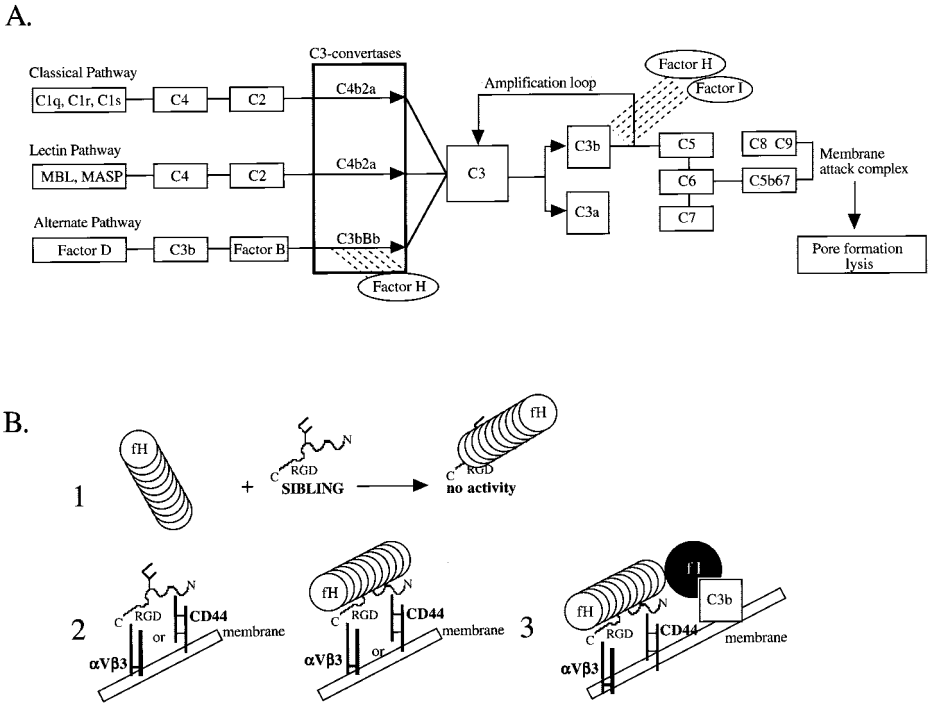

FIG. 9. **Complement pathways and SIBLING activity.** *A*, classical complement involves an antigen-antibody priming event that leads to the recruitment of multiple proteins that ultimately lead to, among other things, lysis of attacked cells. The lectin and alternative complement pathways involve initiation by specific proteins or carbohydrate moieties that cause protein complexes to form and eventually cell lysis. The three pathways converge in the formation of a C3 convertase. All subsequent steps leading to opsonization are shared between the pathways. *B*, model of SIBLING activity. SIBLINGs bound by Factor H (*fH*) in solution cannot subsequently bind to cell surface receptors and have no protective activity against complement-mediated lysis (*1*). Secreted SIBLINGs that bind to nearby cell surface receptors ($\alpha_V\beta_3$ or CD44) sequester Factor H to the membrane surface (*2*). The receptor-SIBLING-Factor H complex posses MCP-like activity in promoting Factor I cleavage of C3b (*3*). *MBL*, mannan-binding lectin; *MASP*, serine protease.

SIBLINGs and prevents systemic SIBLING-mediated complement regulation. From a biochemical standpoint, the significance of the serum complex is that it is necessary to disrupt the complex in order to measure total serum SIBLING levels. We have recently described the development of competitive immunoassays to measure total BSP and OPN in serum from normal donors and patients with various types of cancer (32). Disruption of the serum complex enabled BSP and OPN to be measured with a high degree of sensitivity and specificity.

The four acidic SIBLINGs (BSP, DMP1, dentin sialophosphoprotein, and OPN) are all often considered to be "matrix" proteins, largely because they accumulate in the mineralized matrices of bones and teeth. With respect to immunolocalization within the matrix, BSP and OPN are the two most extensively studied. Within bone, both appear to be enriched in the areas of *de novo* bone synthesis and the location where bone removal by osteoclasts has halted and a layer of collagen-poor matrix is made immediately prior to the formation of replacement bone (33–35). These areas are called cement (or reversal) lines and are where, first, an old matrix is exposed and then a new bone matrix is formed. One intriguing question is whether the SIBLINGs that accumulate there have any complement related activity. Most of the studies of complement involve understanding how cells such as bacteria or infected host cells are opsonized and/or lysed and how our normal cells escape this process. However, because the first step of the alternative pathway of complement is the spontaneous production of activated C3 and this activated C3 will form covalent linkages with various hydroxyl (OH) and amine (NH) groups on proteins and carbohydrates, it is reasonable to assume that exposed matrices will have activated C3 bound to them. Indeed, the subendothelial extracellular matrix *in vitro* becomes labeled with C3 when the lining endothelial cells retract (36). In the case of the mineralized matrices, it is also known that hydroxyapatite is one of the substances that can directly activate the classical pathway of complement (without the contribution of immunoglobulins) (23), thus adding another possible method of locally activating the complement cascade in these tissues.

It is widely assumed that the OH and NH groups of matrices are not as good receiving groups for the activated C3 as are bacteria, infected cells etc., and as such do not accumulate large amounts of complement. Even if this is so, the long period of exposure of some matrices to normal complement (some matrices last for many years), and the lack of the membrane-bound DAF and MCP-like activities available to cells would ensure an accumulating level of at least the more stable NH-bound C3. In light of our recent data, it is intriguing to consider that the accumulation of the SIBLINGs at the very sites of old or new matrix may suggest that these proteins are involved in both quenching the ACP on cells in the immediate vicinity of matrix exposure and also, through interaction with Factors H and I, result in the destruction of any C3 accumulating on the local matrices.

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